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ANTIGEN SPECIFIC SUPPRESSION OF T CELL RESPONSES. I. SUPPRESSOR CELLS ARE NOT CYTOTOXIC CELLS.

by

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ABSTRACT

Observations showing that thymus-derived lymphocytes (T cells) can suppress the induction of various cell-mediated immune responses have been reported only recently. T suppressor cells can suppress in an antigen-specific (50-52) or nonspecific (53-57) fashion the *in vitro* generation of cytotoxic T lymphocytes (CTL). Most of the experimental protocols employed in these studies utilize the mixed lymphocyte culture (MLC) technique for the generation and detection of suppressor cells. The MLC reaction, however, also leads to the induction of a high level of CTL. This observation has led to the suggestion that suppressor T cells are cytotoxic cells and that they may inhibit the induction of CTL by attacking the stimulatory cells (52). On the other hand, other investigators (50, 51, 54) have suggested that some models of suppression of CTL induction cannot be adequately explained as due to elimination of stimulator cells.

The present studies were undertaken to investigate the nature and mechanism of action of suppressor T cells, obtained from primary three-day MLC (first step culture), on the induction of CTL in a secondary MLC (second step culture). The results show that suppressor T cells and cytotoxic T cells are different cells.

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> It is a hazardous world; it has always been a hazardous world, beset by the perils of harsh nature and the greater perils of harsh men. Ambition and cunning and the ignorance of multitudes have created rigid systems that have suppressed all liberties, and from these, men have broken away into freedom at times, have become confused in their councils, and have again succumbed. This time there is hope, for free men have at last created a democracy more effective, as long as it retains its hallmark, than any dictatorship can ever be in dealing with the intricacies of civilization.
> -Vannevar Bush, Modern Arms and Free Men, Simon and Schuster, Inc.

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TABLE OF CONTENTS

		PAGE
	LIBRARY RELEASE FORM	i
	TITLE PAGE	ii
	APPROVAL PAGE	iii
	ABSTRACT	iv
	ACKNOWLEDGEMENTS	v
	TABLE OF CONTENTS	vi
	LIST OF TABLES	viii
	LIST OF FIGURES	ix
Chapter I	Introduction	1
	A. The immune system	1
	B. The immune response	5
Chapter II	Review of the Literature	8
	A. Suppressor cells in humoral immunity	9
	1. Low zone paralysis	9
	2. Suppressor T cells in genetic nonresponder mice	2
	3. Suppression of immune responses to T-cell independent antigen	13
	4. Chronic allotype suppression	14
	B. Suppressor cells in cell-mediated immunity	17
	1. Suppression of DTH responses	17
	2. Suppression of cytotoxic T lymphocytes responses by suppressor T cells	19
	C. A unifying concept of the mechanism and nature of suppressor cell activity	24

PAGE

Chapter III. The Nature and Characteristics of an Antigen-Specific Suppression of the Induction of Cytotoxic Responses 31

A. Materials 31

1. Animals 31

2. Cell lines 31

3. Tissue culture medium 31

4. Tissue culture vessels 31

B. Methods 33

1. The in vitro generation and assay for suppressor cells 33

2. Cytotoxicity assay 34

3. Treatment with hydrocortisone 34

4. Preparation of anti-θ serum and complement . 35

C. Results 36

1. The effect of irradiated cells from first step cultures on the generation of cytotoxic T lymphocytes 36

2. The effect of suppressor cells on the kinetics of the cytotoxic response to Balb/c allo-antigen 36

3. Suppressor cells are T cells 39

4. Absence of an allogeneic barrier to suppressive activity 39

5. Kinetics of development of suppressor cells . 39

6. The effect of in vivo treatment with hydrocortisone on the subsequent in vitro generation of suppressor cells and cytotoxic cells 43

7. Mechanism of action of suppressor cells . . . 47

Chapter IV. Discussion 51

Chapter V. Summary 55

LIST OF TABLES

Table	Description	Page
I	Inhibitory cells as described by Bretscher	28
II	Anti-θ treatment of cells from first step culture	40
III	Strain specificity of the suppressor cell	41

LIST OF FIGURES

Figure	Description	Page
1.	The relationship between the generation of helper activity and the antigen concentration	25
2.	The experimental protocol	33
3.	Generation of antigen specific suppressor cells	37
4.	The effect of suppressor cells on the kinetics of the cytotoxic response	38
5.	Relationship between time of culture incubation and the development of suppressor cells	42
6.	Effect of cortisone treatment on the generation of suppressor cells	44
7.	The effect of first step cultures from cortisone-treated mice on suppressive activity of first step cultures from normal spleen	46
8.	Suppressor cells and responder precursor cells recognize physically linked determinants on the stimulator cell	49

Chapter I. Introduction

A. The immune system

The immune system is a complex, integrated, and delicately balanced multicellular system whose activation is determined by a variety of cellular interactions and sophisticated regulatory mechanisms. These mechanisms form the basis of a remarkably versatile adaptive process which constitutes the principal means of defense of the individual against an immense variety of organic molecules, viruses, and pathogenic microorganisms.

Adaptive immunological responses are only encountered in vertebrates. Lower forms of life do not possess such a sophisticated system of defense. Nevertheless, "non-specific" immune mechanisms of recognition and phagocytosis exist in such species. It is possible, however, that the immune response evolved under selective pressures which could have operated to a considerable extent to increase the efficiency of the non-specific immune system. One way in which the immune response becomes efficient is to increase its library of recognition units. Before this could happen, however, the immune system must acquire the ability to discriminate between foreign "nonself" and "self" antigens. The induction of self-reactive lymphocytes could lead to the production of autoimmunity which is destructive to the individual's own components. In principle, this could prove to be highly embarrassing. Therefore, a mechanism by which the expression of autoimmunity is prevented must exist. One theory suggests that the individual does not have the genetic capacity to synthesize recognition molecules able to bind self-antigens. Another type of theory suggests that the individual has such a capacity but that differentiation of precursor cells which bear anti-self recognition units is prevented. This

implies a mechanism in which self-nonself discrimination is learned. Burnet (1) postulated that those circulating body components which were able to reach the developing lymphoid system in the perinatal period could in some way be learned as self and these cells bearing receptors for self antigens could then be eliminated. An immunological unresponsiveness, "self tolerance"[1], would then be established so that when immunological maturity was reached there would be an inability to respond to self components.

The theory that self tolerance is learned, not inherited, is widely accepted and its predictions have been amply verified by both experimental evidence and theoretical considerations. Experimental studies (2) have demonstrated that, under certain circumstances, autoantibodies can be induced which are able to bind to and damage self components. On purely theoretical grounds, there are two arguments for having a learning mechanism of self-nonself discrimination in the immune system. Firstly, consider the homozygous individuals AA and BB. These individuals have the ability to recognize and respond to each other's antigens. The fact that the F1 hybrid animal AB exists in spite of the presence of genes that allow A to recognize B and B to recognize A suggests that the mechanism for maintaining the integrity of the F1 hybrids is a learned mechanism. Secondly, a genetically determined self-nonself discrimination mechanism would interfere with the evolution of many self components.

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[1] In this thesis, use of the word tolerance will be restricted to mean the natural process by which an animal tolerates or fails to react to its own genetically proper substance. For those experiments carried out in adult animals in which, by certain manipulations, the animals fail to respond to some standard antigenic stimulus, the word "unresponsiveness" will be used. It is a matter of convenience and importance to consider the two conditions of natural tolerance and experimentally produced unresponsiveness separately (1).

For example, newly arising advantageous mutants of body components would be recognized as foreign and consequently would be eliminated. Such a system would be very dangerous to the survival of the species and in evolutionary terms, this type of mechanism could not be allowed to happen. Therefore, a learned mechanism for self-nonself discrimination is favored over a genetic one because it does not interfere with the development and generation of diversity of various body components which is beneficial for the survival of the species.

The ability to respond predominantly against foreign as opposed to self antigens forms the basis of the specificity of the immune system. To be effective the specificity of recognition should go even further. The immune system must also recognize different forms of foreign antigens and respond against each one independently. For example, the response against one organism should not interfere with the response against another unrelated organism. In fact, the immune system can specifically and effectively discriminate between the two organisms.

Another way in which the immune system became more efficient as a means of defense was to develop a mechanism by which the body is effectively prepared to repel strongly any repeated invasion by the same kind of foreign antigen. This mechanism is manifested in the development of "memory" cells. The first contact with an antigen clearly imprints some information, memory, so that a second contact with the same antigen results in the generation of a brisk and strong immunological response. The first response elicited by an antigen is called the primary response and the second response elicited by the same antigen is called the secondary or "anamnestic" response. In the secondary response, the antibody level rises faster and higher (tenfold or more), and after reaching

its peak, it declines much more slowly than the primary response.

The generation of memory cells is a remarkable feature of the immune response. On theoretical grounds it is a necessary correlate of increased specificity. As the library of recognition units of the primitive system is greatly expanded, such that it is more similar to that of the immune system, the frequency of recognition units specific for a certain type of antigen decreases. This in turn allows for a reduction in the efficiency of the immune system. In order to avoid such a dangerous pitfall, upon stimulation with antigen, a specific recognition unit must divide and expand into a clone of progeny cells representing the "memory" of the occurrence. This increases the frequency of cells able to respond to common pathogens. These progeny cells revert to a resting state and are committed to respond strongly if the same antigen should reappear.

B. The immune response

Recognition of an antigen by an immunocompetent lymphocyte is the first step in an immune response. Following this step, a variety of cellular interactions determine whether these cells will be induced or paralysed. Paralysis may involve either specific death and clonal deletion of antigen reactive cells or any other unknown mechanism. The mechanism of clonal deletion was postulated by Burnet (1) as a working hypothesis for the maintenance of self-tolerance. He suggested that newly differentiated immunocompetent cells were destroyed or rendered incapable of multiplication when they recognized and reacted with antigenic determinants of self components. This theory has recently become a subject of debates and various criticisms by many immunologists who favor a mechanism whereby specific immune cells exist whose sole function is to maintain the unresponsive state against self components. Further discussion of this subject is beyond the scope of this thesis and therefore will not be pressed here.

Induction, on the other hand, leads to proliferation and differentiation of antigen reactive cells to immune effector functions. There are two types of immune responses: humoral and cell-mediated. Humoral immunity is effective against bacterial infections and viral infections whereas cell-mediated immunity is most effective in attacks against viral and bacterial infected cells and becomes a problem in its efficiency of attack against foreign tissues, such as transplanted organs.

The division of labor in the immune system occurs through the differentiation of two populations of cells native to lymphoid tissues but also found in other parts of the body, particularly in the blood. These two types of lymphoid cells are morphologically similar and both derive from the same primitive precursors, the hemopoietic stem cells. Stem

cells that develop under the influence of Bursa of Fabricius in birds or the bursa equivalent in mammals (e.g. bone marrow) are called B lymphocytes. These are the cells that give rise to the antibody secreting cells that effect humoral immunity. Cells that develop under the influence of the thymus are called T lymphocytes. This is a large and heterogeneous population of lymphocytes. Some T cells are the effectors of cell-mediated immunity. If these cells are confronted with an antigen they differentiate into immunologically active cells capable of specific killer activity or release of lymphokines that participate in the elimination of the foreign material. The evidence to date suggests that T cells also play a crucial regulatory role in the induction of both humoral and cell-mediated immune responses. This regulatory activity could be viewed in algebraic terms as being either positive or negative. Positive regulation leads to induction and negative regulation results in unresponsiveness "immunosuppression."

The cooperative activity (positive regulation) of T cells involved in the development of antibody and cell-mediated immune responses was the first regulatory function attributed to T cells (3). T cells that function in this manner came to be known as "helper" T cells. The importance of helper T cell activity in the production of antibody responses was best dramatized in the hapten-carrier cell transfer studies of Mitchison (4-6). He demonstrated that the interaction of T cells with antigenic determinants on the carrier molecule led to the production of anti-hapten antibodies by B cells. This observation has been confirmed and extended by Paul (7) and Katz and Benacerraf (8). Similarly, helper activity is also required for the generation of cell-mediated immunity (77).

Negative regulatory mechanisms or immunosuppression, on the other hand, are part of a homeostatic and self-monitoring immune system; that is, recognition of antigen not only stimulates the proliferation and differentiation of responder precursor cells but also stimulates the generation of specific immunosuppressive effects which appear to inhibit the induction of immune responses or to modify an ongoing response. These immunosuppressive effects could be mediated by antibodies, antibody-antigen complexes, or suppressor cells. The concept of antibody and antibody-antigen complexes having inhibitory effects has been extensively reviewed by Uhr and Möller (9), Playfair (10), and Diener (11) and it will not be discussed here. Evidence which suggests that immunosuppressive effects could be attributed to suppressor T cells has accumulated only recently. The nature, characteristics, and mechanism of action of suppressor T cells are the subject of the review which follows.

Chapter II. Review of the Literature

The concept of suppressor cell activity in immune responses was introduced and vigorously championed by Gershon and Kondo (12, 13). They observed that the unresponsiveness of B cells in thymectomized lethally irradiated and bone marrow reconstituted adult CBA mice was dependent on the presence of T cells. The pretreatment of bone marrow reconstituted mice with SRBC in the absence of T cells had no significant effect on the ability of thymocytes to reconstitute the anti-SRBC antibody response. On the other hand, mice which had been reconstituted with a small number of thymocytes at the time of bone marrow reconstitution (prior to the antigen injections) were markedly impaired in their ability to make anti-SRBC antibodies. Following these studies, a number of suppressor cell phenomena were described. In each case, a common property of suppressor cells is that one cell population will inhibit the response or function of a second cell population in a cell transfer or <u>in vitro</u> experiment. The majority of the inhibitory cell populations observed are anti-θ serum sensitive, confirming the T cell dependence of suppressor activity. Furthermore, depending on the mode of action, the suppressive effect can be either antigen specific or nonspecific.

I will now turn to discuss various categories of suppressor T cells in both humoral and cell-mediated immune responses. The evidence on suppression of antibody responses has been extensively reviewed by Katz and Benacerraf (8), Droege (14), and Gershon (15) and therefore only well characterized systems will be discussed here. This review will concentrate on the discussion of available evidence on suppressor T cells in cell-mediated immune responses and the discussion of a unifying

concept with regard to the nature and mechanism of negative regulatory effects referred to "suppression."

A. Suppressor cells in humoral immunity

1) Low zone paralysis

Evidence has been accumulating which indicates that certain patterns of B cell unresponsiveness to a specific antigen do not fit into Burnet's suggested model of deletion of antigen-specific reactive cells. This followed from the observation that T cells obtained from animals made unresponsive against one antigen can, upon transfer to normal syngeneic animals, specifically suppress the antibody response of the host to the same antigen (12, 13, 16). The existence of such a suppressive mechanism, its stability and infectious character on adoptive transfer (17), and the evidence of its potential reversibility (18) suggest that some forms of B cell paralysis can be mediated and maintained by suppressor T cells.

Among the various experimental models of *in vivo* unresponsiveness which have been studied in the past few years, the induced immunological unresponsiveness obtained in mice to the antigen human gamma globulin (HGG) has been the best characterized (19). There are difficulties, however, which overshadow the interpretation and the conflicting nature of experiments which had attempted to demonstrate active suppressive capacity of lymphoid cells obtained from HGG nonresponsive animals. Nevertheless, the critical question appears to be not whether suppression provides mechanistically the *only* means by which specific unresponsiveness is induced and maintained, but whether suppression exists as a supplementary parameter of the state of unresponsiveness.

Evidence showing that suppressive activity exists in the HGG unresponsive system has been extremely convincing. Basten et al. (1974) demonstrated that spleen cells from HGG unresponsive mice abrogated the adoptive secondary response to DNP·HGG of heavily irradiated syngeneic recipients reconstituted with equal numbers of DNP-primed and HGG-primed spleen cells. The suppressive effect was demonstrated to be T cell-dependent and the state of nonresponsiveness could be transferred to normal syngeneic animals. This is consistent with the observation that nonresponsiveness to "T cell-dependent"[2] antigens may be "infectious" (13, 20). The relationship between the induction of the nonresponsive state and suppression was further investigated in three different ways: HGG was deaggregated by (a) ultracentrifugation on an angle-head rotor; (b) ultracentrifugation in a swing-out rotor; and (c) biological filtration (2.5 mg HGG was injected intraperitoneally into normal mice and serum collected from them 24 hours later). In the standard adoptive system, significant suppression was obtained with cells from animals pretreated with HGG deaggregated in the angle-head rotor or biological filtration. In contrast, cells from mice given HGG deaggregated in the swing-out buckets, although were unresponsive on their own, exhibited much less effective suppression. The reason for this discrepancy between preparations has not been resolved. Nevertheless, these results confirm the existence of suppressor cells in the HGG induced state of nonresponsiveness.

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[2] "T cell-dependent" or "T-dependent" antigens refer to antigens which, under standard conditions, require the helper function of T cells to produce a normal IgG antibody response to that antigen (19).

Similar results were obtained by Kolch and Weber (21). They observed that pretreatment of adult CBA mice with low doses of phage fd led to a specific suppression of the anti-phage fd IgG antibody response. The IgM antibody response was not affected. Injection of normal spleen cells did not restore the immune reactivity of the unresponsive mice. Further analysis of the cellular basis of this unresponsiveness involved experiments in which thymus cells were educated with antigen (phage fd) in thymectomized, lethally irradiated mice reconstituted with fetal liver and normal syngeneic thymus cells. They found that educated thymocytes, upon transfer into normal syngeneic mice, inhibited the ability of these mice to respond to immunogenic doses of antigen. The inhibition was antigen specific and dependent on the presence of viable suppressor T cells, since treatment with anti-θ serum and complement abolished transferrable suppressive activity.

2) Suppressor T cells in genetic nonresponder mice

Early studies (22) demonstrated that the antibody response by inbred strains of mice to the random terpolymer of L-glutamic acid-L-alanine-L-tyrosine (GAT) was controlled by an Ir gene which maps in the I region of the H-2 complex. Some strains behave as responder mice, synthesizing specific anti-GAT antibodies and others behave as non-responder mice synthesizing no detectable antibodies after injection of GAT (23). Both responder and non-responder mice, however, produced GAT-specific antibodies when immunized with GAT complexed to the immunogenic carrier methylated bovine serum albumin (GAT-MBSA). This indicates that non-responder mice, like responder mice, could synthesize GAT-specific antibodies if the appropriate T cell helper function is provided. Gershon et al. (24) were the first to show that non-responder

mice were not completely deficient of T cells specific for GAT on the grounds that GAT stimulated the incorporation of thymidine by non-responder spleen cells. They further suggested that genetic non-responders unable to form detectable immune responses to GAT might nevertheless be able to generate suppressor T cells upon immunization with such an antigen. Strikingly enough, Kapp et al. (25, 26) showed that the presence of spleen cells from non-responder mice that have been previously immunized with GAT, inhibited the antibody response of normal non-responder spleen cells to the GAT conjugate (GAT-MBSA). They further demonstrated that this inhibition was due to the presence of GAT-specific suppressor T cells. Extracts prepared from spleen cells and thymocytes of GAT-primed mice were also suppressive. The suppression was antigen specific in that spleen cells from GAT-primed animals did not inhibit the anti-SRBC PFC response by normal non-responder spleen cells even when incubated with SRBC plus GAT-MBSA.

The inability to develop immune response to an antigen, however, cannot in all cases be attributed to the generation of suppressor cells as originally suggested by Gershon et al. (24). Debré et al. (27) demonstrated that the co-polymer of L-glutamic acid and L-tyrosine (GT) did not stimulate the production of suppressor T cells in all the non-responder strains tested. A/J ($H\text{-}2^a$) mice were not suppressed by the injection with GT, whereas SJL ($H\text{-}2^s$) mice were suppressed by the same immunization. The coisogenic strain A.SW which bears the same H-2 haplotype of SJL on the A/J background was suppressed by GT immunization. This observation is in accord with the hypothesis that the control of the generation of suppressor cells is linked to the H-2 complex in a manner analogous to the control of immune responses by H-linked Ir

genes (28, 29). This view is greatly strengthened by the striking similarity between the antigen specific suppressor factor extracted from suppressor T cells by Tada et al. (30) and the antigen specific inhibitory T cell factor recently described by Benacerraf (29). Both factors have specificity and affinity for antigen and contain gene products coded for in the I region of the H-2 complex where Ir gene functions has been mapped.

3) Suppression of immune responses to T cell-independent antigen

In the examples so far, the activities suppressed were B cell responses to T cell-dependent antigens. Suppressor T cell activity for a T cell-independent antibody response against Type III pneumococcal polysaccharide (SSS-III) has been reported by Baker et al. (31, 32). Although helper T cells are thought to be not required for a normal antibody response to SSS-III, the response is greatly influenced by the activities of other types of T cells. These cells were given the term amplifier T cells since their role appears to be to regulate, rather than to initiate, antibody formation (33). The response to SSS-III is predominantly IgM antibodies and treatment with anti-lymphocyte (ALS) or anti-thymocyte (ATS) serum at the time of immunization resulted in a significant increase in the magnitude of the antibody response. Further analysis on the effect of treatment with various amounts of ALS on the magnitude of the IgM response to SSS-III was assessed in both athymic nude and thymus-bearing littermate control mice. After treatment, thymus-bearing mice showed a significant enhancement in the magnitude of IgM plaque-forming cell to SSS-III. In contrast, no enhancement was demonstrable in athymic nude mice given the same test doses of ALS. The authors concluded (a) that ALS-induced enhancement is dependent on

the presence of T cells and (b) that enhancement is not the result of a stimulatory effect of ALS upon B cells.

The foregoing observation is especially important from the viewpoint that low zone paralysis to SSS-III is mediated by activated suppressor T cells which may have developed as a natural consequence of immunization. This possibility was greatly strengthened by the recent findings (33, 34) that treatment with a marginally immunogenic dose of SSS-III specifically reduced the capacity of mice to respond to subsequent immunization with an optimally immunogenic dose of this antigen. Moreover, treatment with either ALS or lactic dehydrogenase virus (LDV) enhanced the response to SSS-III antigen. (Infection with LDV has been reported to result in the cytotoxic degeneration of lymphocytes in the thymus-dependent area in the lymph node and spleen (35). In addition, the ability of ALS or ATS to enhance the antibody response to SSS-III can be removed by absorption with mouse thymocytes but not with nonlymphoid cells.

4) Chronic allotype suppression

Allotype suppression is defined as a phenomenon in which exposure of the fetus or the neonate to antibody against its own immunoglobulins suppresses production of those immunoglobulins (36). Originally, this phenomenon was described in the rabbit (37). A few years later the Herzenbergs reported that allotype suppression could be demonstrated in the mouse. The strains usually used in their studies were Balb/c as maternal or anti-allotype donor which carries Ig-1a and Ig-4a allotypes and C57Bl/10 and SJL/J as paternal strains which carry Ig-1b and Ig-4b allotypes. The corresponding notations for the gene clusters are Ig^a and Ig^b respectively. Jacobson and Herzenberg (38) showed that progeny produced by mating SJL/J (Ig^b) males to Balb/c (Ig^a) females which had

been immunized by Ig-1b allotypes exhibited chronic long-term suppression in which more than one-half of the progeny at six months of age had no detectable Ig-1b in circulation. A key experiment proving that chronically suppressed mice contain an active suppressor cell utilized the mixture-transfer assay. In this experiment, 10^7 spleen cells from suppressed F1 hybrids were mixed in vitro with 10^7 spleen cells from syngeneic normal F1 hybrids and then injected into irradiated Balb/c hosts. Levels of Ig-1b in recipients of cell mixtures were indistinguishable from recipients of 10^7 suppressed cells alone, and significantly below the levels seen in recipients of 10^7 normal F1 hybrid cells. Cells from thymus and lymph nodes were as suppressive as spleen cells whereas bone marrow cells were roughly two-fold better in suppressive activity. Suppression was shown to be due to a thymus-derived cell by various criteria: (1) neonatal thymectomy prevents development of chronic suppression, (2) suppressor cells are killed by treatment with anti-thy-1 and complement, and (3) suppressor cells are not retained on nylon wool columns which retain B-cells but allow T-cells to pass (39). Furthermore, the suppression is specific for Ig-1b production in an Ig^a/Ig^b heterozygote. These observations strongly support the existence of a regulatory suppressor T cell function as a mechanism for chronic allotype suppression.

The mechanism of suppressor cells in chronic allotype suppression is not yet established. One approach to this issue, however, is to focus on the inherent regulatory role of T cells in the differentiation of B cells. Suppressed mice have been shown to have normal levels of Ig-1b memory cells indicating that these mice have reasonably mature precursors of Ig-1b-producing cells (40, 41). The locus of the suppressor cell attack, therefore, is narrowed to the latter part of the B cell differentiation

pathway at a stage very close to the point where cooperator cells interact to facilitate memory cell differentiation. Under normal conditions, cooperators and suppressors exist in a state of a finely tuned balance. The presence of anti-allotype serum in the young animals, however, could shift this balance in favor of overregulation of Ig-1b synthesis. This early shift could result in the establishment of an overdeveloped population of regulator (suppressors) T cells which specifically suppress Ig-1b precursors from differentiating to producers. In fact, the Herzenberg experiments show a stochiometric relationship between helper T cells and suppressor T cells suggesting that suppressors act by antagonizing helper T cells thereby preventing B cell interaction.

B. Suppressor cells in cell-mediated immunity

Observations showing that T cells can suppress the induction of various cell-mediated immune responses have been reported only recently. Suppressor T cells have been shown to suppress such responses as the induction of delayed-type hypersensitivity (42-46), T cell proliferation (15, 47-49), and the development of cytotoxic T lymphocytes (50-54). Con A-induced suppressive activity has been also reported (55-57). Furthermore, suppressor T cells have been implicated in prolongation of allografts in mice (58-60) and in chickens (61). However, the suppressor activities in most of these systems are not sufficiently characterized and, therefore, for the purposes of this thesis, I shall concentrate on a review of suppressor T cells in the regulation of the development of delayed-type hypersensitivity (DTH) and the generation of cytotoxic T lymphocytes (CTL).

1) Suppression of DTH responses

Suppressor T cells have been shown to specifically inhibit the development of DTH responses to such antigens as picryl chloride (43) and dinitroflorobenzene (44,62). Asherson et al. (42) showed that suppression of contact sensitivity can be specifically induced by injection of picrylsolfonic acid prior to painting the ear with a dilute solution of the contactant picryl chloride. Claman et al., using dinitroflorbenzene (DNFB) as a sensitizer, reproduced and extended the work of Asherson and his colleagues. Mice, sensitized by two paintings with 0.5% DNFB on the abdomen, were challenged after 5 days by painting the ears with 0.5% DNFB. 24 hours later ear swelling was measured with a micrometer. Mice previously treated with $DNBSO_3Na$ failed to show ear swelling as compared to non-treated mice. Unresponsiveness induced by

this method was shown to be specific and can be transferred, using unresponsive spleen or lymph node cells, to normal mice. Serum from suppressed mice failed to induce unresponsiveness upon transfer to normal mice. Depletion of B cells by passing over an anti-Ig column did not abrogate the ability of cells from unresponsive mice to inhibit the induction of DTH in normal mice. Cells treated with anti-θ serum and complement failed to transfer suppression of DTH to normal mice which indicates that the inhibition of contact sensitivity is mediated by suppressor T cells.

In a different system, similar results were obtained by Ramshaw et al. (46). They observed that the transfer of spleen cells or Ig negative (Ig^-) spleen cells from mice immunized to produce a humoral response completely suppressed the development of DTH in mice which had been treated with cyclophosphamide prior to cell transfer. (Mice treated with cyclophosphamide, before an optimum sensitizing dose of antigen, exhibit an enhanced DTH reaction.) DTH was measured as footpad swelling. After two days of treatment with cyclophosphamide, mice injected intravenously with HRBC produced a strong DTH reaction to HRBC injected into the footpad. The development of DTH in such mice was suppressed by adoptively transferring Ig negative spleen cells from normal mice which had been injected intravenously with 10^9 HRBC; administration of such a dose of antigen produced high levels of humoral immunity but no detectable DTH. Immune serum, hyperimmune serum or serum from mice whose DTH was suppressed by the transfer of immune spleen cells had no effect on the induction of DTH. This indicates that antibodies do not play any significant role in the suppression of DTH. Furthermore, the suppression was shown to be antigen specific and dependent

on the presence of T cells.

The observation that the establishment of one type of immunity actively suppresses the development of other forms of immunity bears a great deal of significance with regard to the nature and mechanism of suppressor cells. Bretscher (63) has predicted that the inverse relationship between humoral and cell-mediated immunity forms the basis of regulation of the immune response. This model will be expanded upon in the discussion of a unifying concept of the nature and mechanism of regulatory activity.

2) Suppression of cytotoxic T lymphocytes responses by suppressor T cells

A number of workers have shown that suppressor T cells can suppress in an antigen specific (51, 52) or nonspecific (53, 54) fashion both in vitro (49, 51-54) and in vivo (50) generation of cytotoxic T lymphocytes (CTL). These studies have been concerned with (1) the method of sensitization most apt to stimulate suppressor T cells and their distribution in lymphoid tissues; (2) the specificity of suppressor cells; (3) the target of suppressor activity; (4) the mechanism of action of suppressor cells. Most experimental systems utilized the mixed lymphocyte culture reaction (MLC) for the generation and detection of suppressor cells. The one way MLC reaction employed for the generation of suppressor cells, usually referred to as primary MLC, consists of two allogeneic cell populations: responders and stimulators. The stimulator cells are inactivated by treatment with either mitomycin C or x-irradiation. At the end of a given period of incubation, the cells are harvested, washed, irradiated and added to a fresh one way MLC which is referred to as secondary MLC. The time of incubation and

the dose of irradiation depend on the investigator. The secondary MLC is employed for the detection of inhibitory activity that may have been generated in the primary MLC on the development of CTL. The secondary culture, therefore, consists of three cell populations: responder, stimulator, and regulator (cells from primary MLC). Usually, the regulatory cells are syngeneic to the responder cells and the stimulator cells bear the same H-2 haplotype in both primary and secondary cultures.

The fact that the MLC reaction is utilized to generate high levels of CTL has led to contradictory interpretations of the nature and mechanism of the regulatory cells induced in the primary MLC. A cell-mediated cytotoxic interaction between suppressor cells and stimulator cells has been postulated as the underlying mechanism for the suppression of both proliferative (48) and cytotoxic responses in MLC (52). On the other hand, several investigators have suggested that some models of suppression of CTL induction (51, 54, 64) and proliferation in MLC (49) cannot be adequately explained as due to elimination of stimulator cells.

Investigators ascribing suppressor activity to cytotoxic mechanisms employed experimental conditions under which high cytotoxic activities are generated. For example, Fitch et al. (52) observed that cells obtained from five-day primary MLC, the optimum time for generating peak CTL, completely inhibited the generation of CTL in vitro. The inhibitory activity was antigen specific and was directed toward the alloantigen against which the cells from primary MLC were cytotoxic. They showed that the presence of primary MLC cells having cytotoxic activity toward one alloantigen abolished the response to another non-cross-reacting alloantigen only when both antigens were present on the same F1 hybrid stimulating cells. Only the response to the sensitizing alloantigen was

inhibited in cultures where a mixture of the two non-cross-reacting alloantigens were used as stimulator cells. In view of these observations, the authors concluded that the suppression of generation of CTL by irradiated cells from primary MLC involves inactivation of the alloantigen-bearing stimulating cells as a result of residual cytotoxic activity of the primary MLC cells.

The foregoing hypothesis has much to recommend it in that it assigns a role to suppressor cells consistent with a known T cell function, cytotoxicity. If suppressor cells were cytotoxic cells and therefore suppression of generation of CTL were due to elimination of stimulator cells, then one would predict that: (1) pre-incubation of stimulators and suppressors prior to initiation of secondary cultures should increase the degree of suppression; (2) increasing the numbers of stimulator cells at initiation of secondary cultures should competitively reduce suppressor cells activity. The data, however, does not support these predictions. Sinclair et al. (65) have shown that pre-incubation of cells from five-day primary MLC cultures with stimulator cells prior to the addition of responder cells gave less suppression of the generation of CTL in the secondary MLC than pre-incubation of primary MLC cells with responder cells. In fact, the latter gave the most profound suppression. Furthermore, altering the number of stimulator cells in the secondary culture did not influence the degree of suppression (51), whereas varying the concentration of responder cells had the greatest effect on the degree of suppression: strong suppression at low numbers of responders and weak suppression at high numbers of responder cells.

Similar results were reported by Truitt et al. (64). In these studies, in vivo stimulated spleen cells were used as the source of

regulatory population. Balb/c mice were immunized with C57Bl/6 spleen cells via footpad injection. Irradiated spleen cells, obtained from these mice four days after immunization, suppressed the generation of CTL by MLC prepared with Balb/c responder cells and C57Bl/6 stimulator (irradiated) cells. The results from further experiments indicated that increasing stimulator cell concentration in MLC did not competitively diminish the suppressor activity; rather, the magnitude of suppression increased as the stimulator cell concentration was increased.

Further evidence showing that suppressor cells are separable from cytotoxic cells comes from studies in vivo. Cells from allosensitized mice exhibited weak cytotoxic activity when tested immediately after removal from the animal (50). Yet, after a short (48-72 h) period of incubation in vitro, they generated strong cytotoxic activity. Treatment of these cells with mitomycin C blocked their ability to develop into cytotoxic cells. In view of these findings, an attempt was made to separate the cells responsible for the suppressive effect from those having the potential to differentiate in vitro into CTL. Regulatory cell populations from alloimmunized mice were subjected to velocity sedimentation at 1xg. The individual fractions were then tested for prekiller activity and for suppressor activity. The results indicated that, on the basis of cell size, suppressor cell precursors were clearly separable from cytotoxic precursor cells (50).

Another approach to the problem of dissociating suppressor activity from cytotoxic activity was investigated by Hirano and Nordin (54). Their system is slightly different from the others insofar that unirradiated cells from two-day MLC were used as source of suppressor cells. They found that cells from two-day primary MLC prepared with responder

cells from normal mice can develop a high level of cytotoxicity and can nonspecifically suppress the generation of CTL in secondary MLC. In contrast, cells from two-day primary MLC prepared with responder cells from cortisone-treated animals can develop a high level of cytotoxicity but failed to suppress the generation of CTL in secondary MLC. Although these results are not conclusive, they are suggestive of the possibility that suppressor cells (cortisone sensitive) are different from cytotoxic cells (cortisone resistant).

The interpretation that the observed suppression is a result of cytotoxic interaction between stimulators and suppressors does not account for all the experimental results previously described. A more likely interpretation of data showing altered responses induced by a regulator cell population is to consider the possible interactions of all three cell populations in vitro: responders, stimulators, and suppressors. The suppressor cells may either interact with stimulator cells, with responder cell precursors, or with both simultaneously. This interaction could be a direct cell to cell interaction or it could be mediated either by a factor elaborated by the suppressor or other cells activated by the suppressor. A noncytotoxic, H-2 restricted interaction between suppressor and responder cells mediated by soluble factor which suppresses proliferation in MLC has been described (49). Results from the experiments described above also reflected on interaction between the alloantigen-activated suppressor cells and syngeneic responder cells. This suggests that the observed suppression of the generation of CTL in secondary cultures could be due to a regulatory interaction different from cytotoxicity. This view is strongly favored by the evidence presented in this thesis.

C. A unifying concept of the mechanism and nature of suppressor cell activity

There is not much data at present as to what exactly happens at the level of the cellular mechanisms involved in immunosuppression. However, a symposium on suppressor cells in immunity held in London, Ontario, took on the task of bringing together some useful tools by which suppressor cells and their targets could be defined. One of these tools was presented by Cantor et al. (66) who showed that peripheral thy-1^+ cells can be divided into three subclasses: Ly-1^+, Ly-$2,3^+$, and Ly-$1,2,3^+$. While Ly-1^+ and Ly-$2,3^+$ cells are resistant to the short-term effects of adult thymectomy, Ly-$1,2,3^+$ are sensitive to these effects. This suggests that Ly-$1,2,3^+$ develop earlier in ontogeny than Ly-1^+ and Ly-$2,3^+$. Furthermore, the authors demonstrated that depletion of Ly-1^+ cells abolished the subsequent helper activity to SRBC in adoptive syngeneic irradiated hosts. On the other hand, depletion of Ly-$2,3^+$ abolished the generation of cytotoxic lymphocytes both in vivo, in irradiated F1 hosts, and in vitro (in 5 days MLC). This suggests that the Ly-1,2,3 antigens could serve to distinguish different T cell functions. Based on these results, the authors raised the following question: Is suppressor cell activity confined to a differentiated subline of T-cells, distinct from or the same as other subclasses of T-cell? Separation and characterization of this subclass of cells and its products would then permit definitive studies of the cellular mechanisms underlying T-cell suppression. With respect to the Ly markers, evidence to date suggests that there are two types of suppressor T cells. The suppressor T cells of cell-mediated immunity (DTH) are Ly-1^+ and Ly-2^- (46), whereas the suppressor cells of humoral immunity are Ly-1^- and Ly-2^+ (72). This observation would be best explained within the concept

of the immune class regulation theory (63). This theory is based on the self-nonself discrimination theory which has been discussed elsewhere (67, 68). Briefly, the theory states that different classes of precursor cells require different levels of helper cells to be induced. Precursor cells for CMI require low levels of helper activity and precursor cells for humoral immunity require higher levels of helper activity. The theory also states that high levels of helper activity suppress the induction of CMI. The amount of helper activity induced is dependent on the concentration and the antigenicity or "foreignness" of the antigen. This relationship is expressed in Figure 1. The

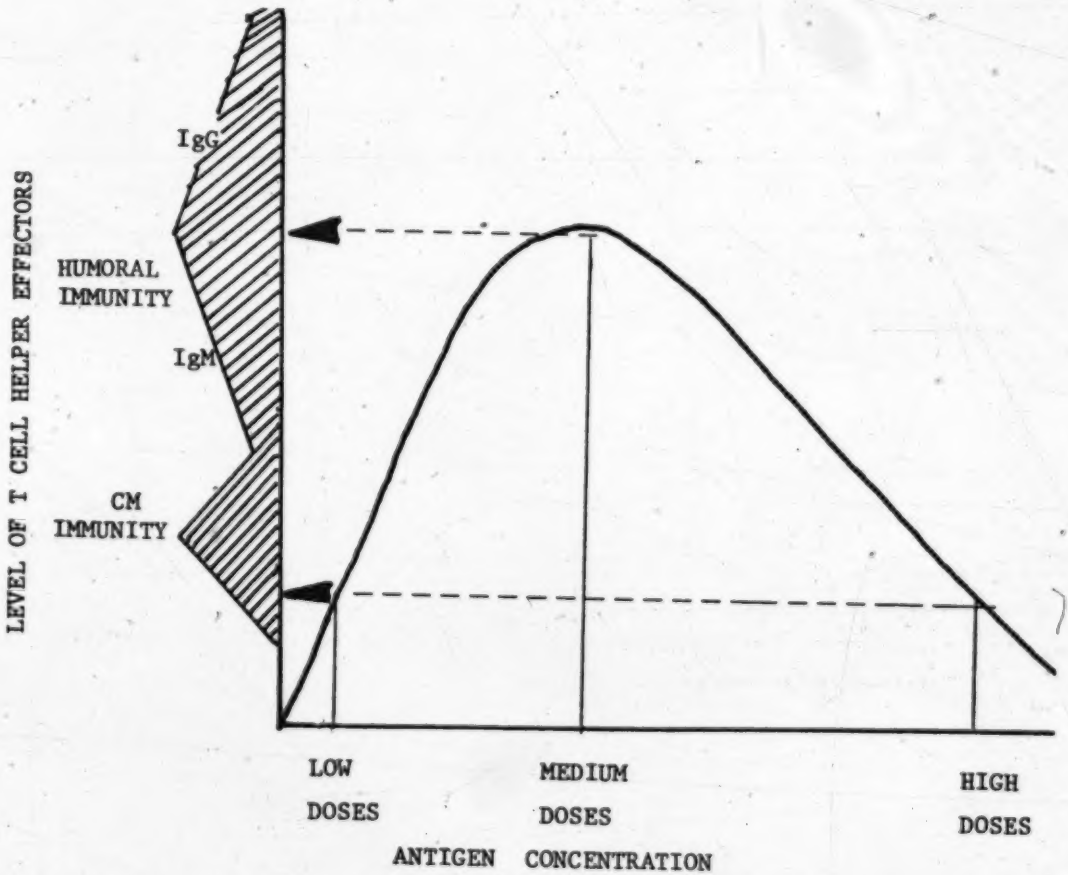

Figure 1. The relationship between the generation of helper activity and the antigen concentration. Reproduced from the original figure by permission from Dr. P. Bretscher (63).

precursors for helper T cell effectors are induced at all but most extreme values of antigen. Low and high concentrations of antigen result in the generation of low helper activity and therefore only CMI is induced. As the antigen concentration increases (i.e. at a medium dose of antigen), maximum helper activity is generated and humoral immunity is induced whereas CMI is suppressed. Similarly, an antigen with few foreign sites induces low levels of helper activity and only generates CMI while an antigen with many foreign sites induces high levels of helper activity which results in the induction of humoral immunity and suppression of CMI. Since a shift to a high level of helper activity, sufficient to induce a humoral response, can suppress the induction of CMI, and since a physiologically effective immune response must have the potential to maintain stable CMI, a regulatory mechanism must be expressed to inhibit the induction of helper activity. According to the theory, this mechanism could be mediated by a cell-mediated effector "antibody," or an antigen specific molecule co-ordinately expressed with CMI which acts on the helper cell precursors. This inhibition is referred to as repression and is maintained as long as a cell-mediated immune response is required. The concept that too much help can suppress the induction of CMI predicts that cells from animals which exhibit an ongoing humoral response should suppress the induction of CMI to the same antigen in a normal syngeneic animal. This prediction has been verified by Ramshaw et al. (46) who demonstrated that HRBC-specific T cells from mice expressing humoral immunity were able to suppress the induction of HRBC-specific DTH. Suppressor cells in this system were shown to be Ly-1$^+$ cells (69). Since helper T cells are also Ly-1$^+$, the mechanism of this suppressive activity has been

postulated to be due to the presence of high levels of specific helper T cells.

The regulatory mechanisms proposed by this theory are more attractive in the sense that it can account for the mechanism of inhibition of an antibody response in some systems. Thus the discrepancy in the results between preparations of human gamma globulin (HGG) deaggregated in a swing-out head centrifuge and HGG deaggregated in an angle-head rotor could be explained as follows. Deaggregated HGG (dHGG) in a swing-out bucket results in paralysis only (19). Mixing studies have shown that dHGG-treated mice have a deficiency of both B cells and precursors of helper cell effectors specific for HGG (70). The unresponsive state of these mice could be due to clonal deletion of precursors for all classes of immunity and is analogous, according to the theory, to the normal state of the immune system to self components (63). On the other hand, mice treated with dHGG prepared in an angle-head rotor results in "repression" (19). The angle-head preparations could be slightly immunogenic and therefore capable of inducing CMI which results in the repression of the antibody response to dHGG. Early studies demonstrated that the unresponsiveness in such mice is similar to that of "low zone paralysis." Another example of "low zone paralysis" is the unresponsiveness to phage fd antigens (21). In most of these systems, the inhibitory cells are theta-bearing cells. According to the theory, this inhibitory effect could be due to the development of CMI and the theory predicts that such mice should have CMI to the antigen.

Similar conclusions can be drawn for the nonresponsiveness to GAT (26). Non-responder mice, primed with GAT, should exhibit cell-mediated immunity to GAT. Consequently, the induction of cell-mediated immunity will tend to <u>repress</u> the induction of any helper T cell precursors specific

for GAT or any epitope on or linked to GAT (63). Thus, in the presence of GAT-primed non-responder cells (repressors), the GAT-carrier conjugate is less able to induce specific helper T cell effectors and hence the presence of these cells inhibits the humoral response.

The above discussion of immunoregulation as proposed by Bretscher's concept of immune class regulation is summarized in Table I. Repressors are generated concomitantly with the induction of CMI, carry the Ly 2 marker, and can inhibit the development of humoral immunity by acting on the precursors of helper T cell effectors. Suppressors are induced with the humoral response, carry the Ly 1 marker, and inhibit the development of CMI by acting on the CMI precursors.

Table I. Inhibitory cells as described by Bretscher.

Type of inhibitor	Ly marker	Class of immunity inhibited	Induced concomitantly with	Predicted mode of action
Repressors	Ly 2^+	humoral	CMI	inhibits generation of help
Suppressors	Ly 1^+	CMI	humoral	inhibits induction of CMI precursors

Another approach to the search for the mechanism of immunoregulation is to define the target of the inhibitory activity. As postulated above, helper T cell precursors are good candidates as targets for repressors and precursors of CMI are good candidates as targets for suppressors. Evidence suggesting that inhibitory T cells can inhibit the generation of helper T cell activity has been reported by Hamaoka et al. (78). Further evidence indicates that the suppressor activity, which inhibits the induction of CTL, is directed against the responder cell population (51). This, of course, could be the precursors for helper cell activity

or the precursors for the generation of CTL. Whichever might be the case, this indicates that T-T cell inhibitory interactions are quite possible.

Evidence for a T-B cell suppressor interaction which involved the suppressor cell acting directly on the B cell arose from the observation that suppressor cells can inhibit the response to thymus-independent antigens (33). This argument, of course, is difficult to circumvent because a thymus-independent antigen may be one in which only B cells are required or one in which a very small number of T cells are also required. Direct evidence in support of T-B cell inhibitory interaction comes from the work of Basten et al. (19) who showed that the site of suppression in the inhibition of the anti-DNP·HGG response is the hapten-sensitive precursor cell (B cells). These findings, however, do not indicate that the suppressor T cells are acting on the B cells alone. The possibility of the involvement of macrophages in this inhibitory interaction cannot be ruled out.

Macrophages are also good candidates as targets for the inhibitory activity. Macrophages are known to be involved in almost every sort of immunological response. The possibility that negative regulatory effects may involve direct or indirect macrophage-T or macrophage-B cell interactions was verified by several investigators. Pierce et al. (73) demonstrated that a ConA-stimulated inhibitory activity appeared to act on the splenic macrophages. Another evidence in favor of this comes from the work of Asherson and Zembala (79) and Basten et al. (19) on the cells mediating the suppressor effect which demonstrated a requirement for adherent cells, probably macrophages. In this case, a macrophage could play either one of two roles. Firstly, a macrophage may receive an inhibitory signal and itself becomes inactivated. This

suggests that the macrophages are the final targets for the inhibitory activity and a loss in their function leads to a loss of the immune response. Secondly, a macrophage may act as a mediator for the suppressive activity and deliver the inhibitory signal to other responding parties, i.e. T cell precursors or B cell precursors. On an experimental basis, it is very difficult to distinguish which of these possibilities is taking place. Nevertheless, the concept that the actual mechanism of the regulatory events which take place in the immune response could involve the macrophages is an important one and demands further verification.

Chapter III. The Nature and Characteristics of an Antigen-specific Suppression of the Induction of Cytotoxic Response

A. Materials

1) Animals

Male and female adults of the inbred strains Balb/c ($H\text{-}2^d$), C3H·SwSn ($H\text{-}2^b$), CBA/CaJ ($H\text{-}2^k$) mice (8 to 12 weeks old) were obtained from the breeding colony at the University of Alberta. (Balb/c x C3H·SwSn)F1 hybrids were bred from Balb/c females and C3H·SwSn males.

2) Cell lines

P815 mastocytoma ($H\text{-}2^d$; derived from DBA/2) and E14 leukemia ($H\text{-}2^b$; derived from C57B1/6) were maintained in *in vitro* culture conditions. P815 cells were grown in H-16 (Dulbecco's modified) and EL4 cells were grown in F-15 medium (Eagles' Minimal Essential Medium).

3) Tissue culture medium

Eagles' Minimal Essential (F-15), Delbecco's Modified (H-16) and Leibovitz Media were obtained in powder form from Grand Island Biological Company (Gibco). All cultures were grown in F-15 supplemented with 10% F.C.S. (Gibco) and 50 µgm/ml Gentamycin, Microbiological Associates. F-15 contained a final concentration of 10^{-4}M mercaptoethanol, 20 µg/ml of penicillin streptomycin, Gibco and 0.8 mM/ml of L. Glutamine, Gibco.

4) Tissue culture vessels

Marbrook polyacrylamide tissue culture vessels (rafts) were prepared according to the method described by Marbrook and Haskill (74) and Pilarski and Borshevsky (77). The bottom half of the inner chamber of the raft is subdivided into 36 individual V-bottom wells and holds a total volume of one ml. This allows for a continuous flow of the supernate medium for all

36 wells in a raft. After they are prepared, the rafts were washed with four overnight changes of saline, autoclaved and then stored at 4°C. The rafts were equilibrated with two overnight changes of medium two days before their use in culture. These rafts float in a 15 x 60 mm petri dish containing four mls of medium and FCS.

B. Methods

1) The in vitro generation and assay of suppressor cells

Suppressor cells were generated in a mixed lymphocyte culture in which 3-5x10^6 responder cells and 5-8x10^6 irradiated stimulator cells were co-cultured in Marbrook acrylamide rafts (Figure 2). Three days later, the cells from these first step cultures were washed, irradiated, and divided into two parts: one part was assayed for cytotoxic activity and the second was added to a fresh MLC, referred to as the second step culture, to determine its suppressive activity. Second step cultures were prepared either in acrylamide rafts or v-bottom microtiter plates (96 wells). 5x10^5 CBA lymph node responder cells were mixed with 16x10^6 irradiated stimulator cells per raft. One-tenth of these cell preparations, unless otherwise specified, were added per well to the microtiter plate. After five days in culture at 37°C and in 10% CO_2 air atmosphere, cytotoxicity was assayed according to the method described below.

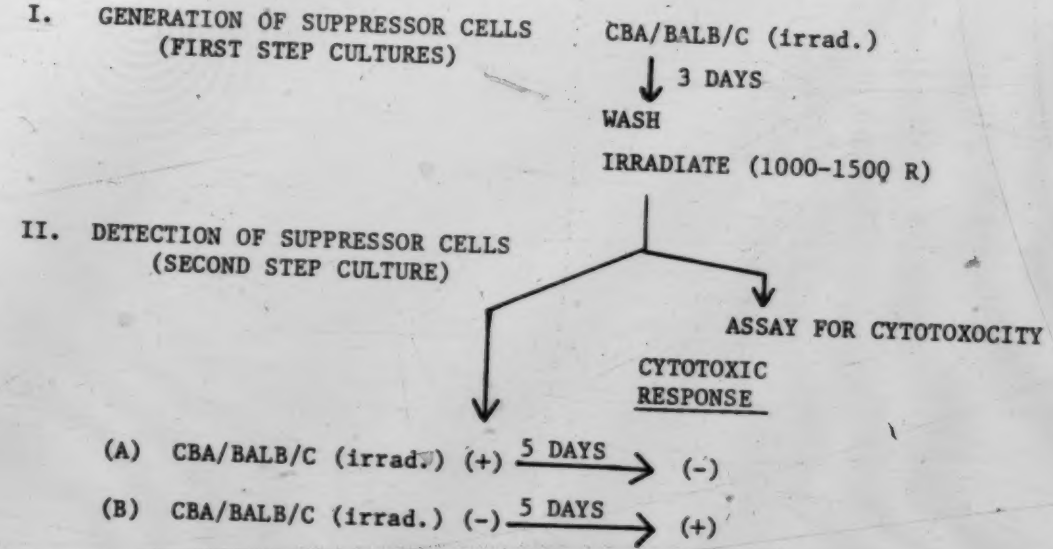
I. GENERATION OF SUPPRESSOR CELLS (FIRST STEP CULTURES)
CBA/BALB/C (irrad.)
3 DAYS
WASH
IRRADIATE (1000-1500 R)
II. DETECTION OF SUPPRESSOR CELLS (SECOND STEP CULTURE)
ASSAY FOR CYTOTOXOCITY
CYTOTOXIC RESPONSE
(A) CBA/BALB/C (irrad.) (+) 5 DAYS (-)
(B) CBA/BALB/C (irrad.) (-) 5 DAYS (+)

Figure 2. This diagram represents the experimental protocol for the generation and detection of suppressor cells.

2) Cytotoxicity assay

The microtiter plates were assayed as follows: the medium in each well was removed and the cells were resuspended in 0.1 ml of fresh culture medium. To each well, 0.1 ml of ^{51}Cr-labelled P815 (10^6/ml) target cells were added. In the rafts the cells were harvested by resuspending each of the 36 pellets/raft into and pipetting the cell suspension into a centrifuge tube. Cells were then washed once and resuspended in one ml of fresh medium and FCS. Several dilutions of those cells were always assayed and all assays were set up in triplicate at each dilution. Each assay consisted of 0.1 ml containing 10^5 ^{51}Cr-labelled target cells and 0.1 ml of cells from each dilution of cultured lymphocytes per well in v-bottom microtiter trays.

In order to determine the total releasable ^{51}Cr, 0.1 ml of 0.05% Triton-x-100 detergent was added to 0.1 ml (10^5 cells) ^{51}Cr-labelled targets. Spontaneous lysis was determined by incubating 0.1 ml of targets with 0.1 ml of medium. These were done in six replicate cultures. Trays for all assays were incubated at 37° in 10% CO_2-air mixture for 4-6 hours. The top 0.1 ml of supernatant from each well was collected in a glass tube and counted in a gamma counter. Per cent specific lysis was calculated as follows:

$$\% \text{ specific lysis} = \frac{\text{sample cpm} - \text{spontaneous cpm}}{\text{detergent lysis cpm}} \times 100$$

3) Treatment with hydrocortisone

Hydrocortisone in acetate suspension (Cortone, Merck Sharp and Dohme), at a dose of 5 mg per animal, was injected subcutaneously. Three days after a single injection of cortisone, the animals were killed and their spleen cells were used in first step culture.

4) Preparation of anti-theta serum and killing of theta-bearing cells

AKR anti-CBA theta serum was prepared by the method of I. Ramshaw (personal communication). AKR mice (2-3 months old) were injected intravenously with $20x10^6$ CBA thymocytes per animal. The thymocytes were obtained from 5-week-old CBA/CaJ mice. A week later, animals were bled and the serum was collected, filtered and stored in one ml aliquots at -70°C. The serum was tested at final dilutions of 1/3, 1/6, and 1/8. The treatment involves incubating cells at $2x10^7$ per ml with the anti-θ serum for 30 minutes in a 37° water bath. Then rabbit complement preabsorbed with mouse tissues was added to a final dilution of 1/15 and the mixture was incubated at 37°C for 45 min. This procedure yielded efficient killing of T cells and no effects on antibody-forming cells or B cells (Pilarski, unpublished). At a final dilution of 1/8 the killing was 83.8% of lymph node T cells, 52.6% of splenic T cells, and 100% of thymus cells.

C. Results

1) The effect of irradiated cells from first step cultures on the generation of CTL

Cells from MLC prepared with $5x10^5$ normal CBA lymph node cells and $16x10^6$ irradiated Balb/c spleen cells showed optimum levels of CTL specific for Balb/c ($H\text{-}2^d$) antigens after five days in culture. This response could be inhibited by the addition of 10^6 irradiated cells from first step cultures of normal CBA lymph node or spleen cells activated against Balb/c antigens. The suppressive effect of first step cultures on the development of CTL is antigen specific, as shown in Figure 3. Irradiated cells from CBA anti-Balb/c first step cultures completely inhibited the cytotoxic response to Balb/c antigens whereas the response to C3H·SwSn ($H\text{-}2^b$) antigens was not affected. Lower numbers of irradiated cells (10^4) from first step cultures reproducibly enhanced the cytotoxic response to both $H\text{-}2^d$ and $H\text{-}2^b$ antigens. This enhancement is likely to be due to cross-reactive activity at the helper cell level, which has been previously observed in this laboratory (Baum and Pilarski, unpublished data). Further evidence for the specificity of the inhibitory activity of first step cells will be described below.

2) The effect of suppressor cells on the kinetics of the cytotoxic response to Balb/c alloantigens

It was possible that irradiated cells from first step cultures may have changed the time required for the generation of optimum level of cytotoxicity in the second culture. Since low numbers of irradiated cells from first step cultures enhanced the cytotoxic response (Figure

3), it could be argued that suppression of the cytotoxic response at day five of culture was actually due to a shift in time course with peak cytotoxicity occurring at an earlier time. We therefore examined the kinetics of the development of CTL in the presence of suppressor cells. Irradiated CBA anti-Balb/c first step cells were added to a second MLC and after a given period of incubation (1,2,3,4,5,7 days) the cultures were assayed for the presence of cytotoxic activity. Figure 4 shows that the peak of the cytotoxic response occurred at day five and that the addition of 10^6 irradiated cells from first step cultures completely suppressed the response at all times tested. This indicates that the suppression we observe is not due to a shift in the kinetics of the cytotoxic response.

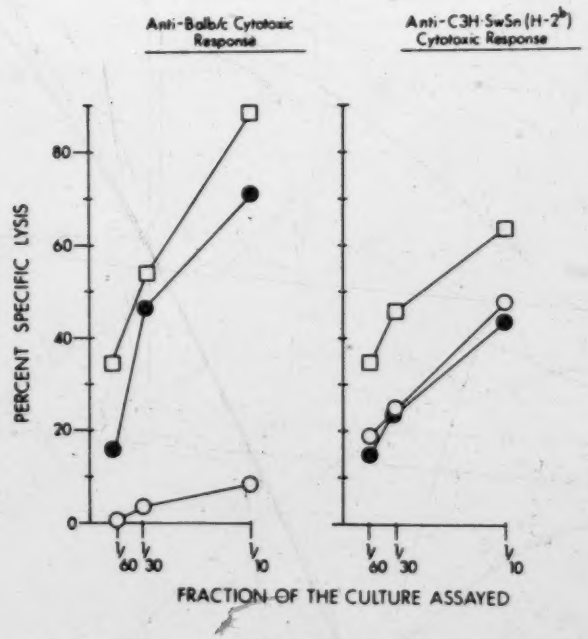

Figure 3. Generation of antigen-specific suppressor cells. After three days in culture, CBA (H-2^k) anti-Balb/c first step cells were irradiated and added, as indicated, to second step culture prepared with $5x10^5$ CBA

Figure 3 cont'd.

LN responder cells and $16x10^6$ irradiated Balb/c (H-2^d) spleen stimulator cells or $16x10^6$ irradiated C3H·SwSn (H-2^b) spleen stimulator cells. After five days in culture, the cytotoxic response was assayed on the corresponding ^{51}Cr-labelled target cell: Anti-(H-2^d) response was assayed on P815 targets, spontaneous release = 829 ± 116 cpm, detergent release = 8253 ± 478 cpm; and anti-H-2^b) response was assayed on E14 targets, spontaneous release = 1547 ± 95 cpm, detergent release = 12269 ± 639 cpm. ●—● No first step cells added; ○—○ 10^6 first step cells added; □—□ 10^4 first step cells added.

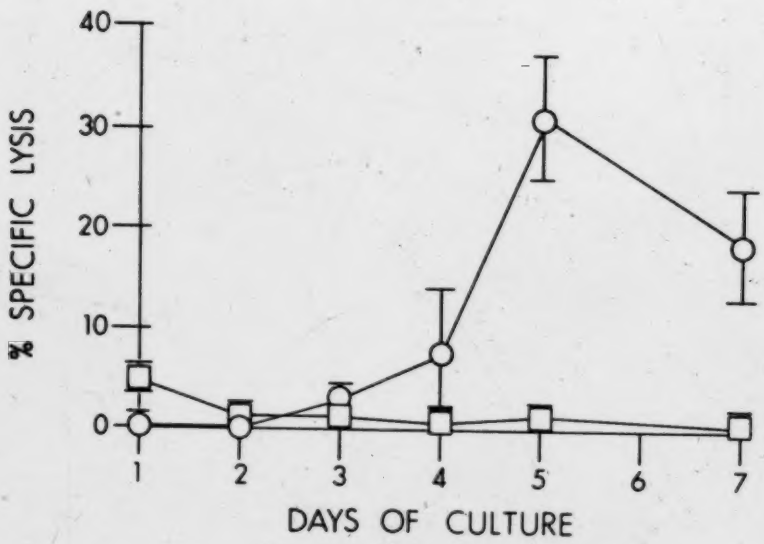

Figure 4. The effect of suppressor cells on the kinetics of the cytotoxic response. Second step cultures were incubated with (□—□) or without (○—○) irradiated CBA anti-Balb/c suppressor cells. After the indicated period of incubation, the cultures were assayed for the presence of cytotoxic activity. Second step cultures were prepared in microtiter trays where $5x10^5$ CBA responder cells, $1x10^6$ irradiated Balb/c stimulator, and $1x10^6$ first step cells were added per well. The results are expressed as the mean cpm ± standard deviation of six replicate cultures.

3) Suppressor cells are T cells

CBA anti-Balb/c cells, harvested on day 3 from a first step culture, were treated with either anti-θ serum and complement or NMS and complement. The cells were washed, resuspended in culture medium and added to a second MLC prepared with CBA lymph node cells and Balb/c irradiated spleen cells. The suppressive effect of the cells from first step cultures is abolished by treatment with anti-θ serum and complement, as shown in Table II. In contrast, untreated and NMS-treated first step cells significantly suppress the generation of CTL.

4) Absence of an allogeneic barrier to suppressive activity

Further studies demonstrated that the suppressor activity is not allogeneically restricted. The results in Table III show that CBA($H\text{-}2^k$) anti-Balb/c first step cells suppressed the anti-Balb/c cytotoxic response of allogeneic C3H·SwSn ($H\text{-}2^b$) lymph node responder cells as effectively as they suppressed the response of syngeneic CBA responder cells to Balb/c alloantigens. This indicates that the suppressive activity of first step cultures does not require H-2 compatibility between responder cells and suppressor cells.

5) Kinetics of development of suppressor cells

During in vitro generation, CTL reach a peak level after five days of culture (Figure 5). In view of this, it is possible that the suppressive effect of irradiated cells from 3-day first step cultures could be due to the appearance of cytotoxic cells in these cultures. An experiment was designed to determine whether suppressor T cells and cytotoxic T cells are always generated under the same conditions. First step cultures were set up on consecutive days and the cells from these cultures were then washed, irradiated and tested in two ways: (a) to assay for

Table II. Anti-θ treatment of cells from first step culture

	Irradiated first step culture	Cytotoxic response Experiment 1 Culture dilutions 1/10	1/30	1/60	Experiment 2
1.	-	4260 ± 143[a] (33.4)[b]	2545 ± 44 (15.2)	2006 ± 9.4 (9.4)	7203 ± 389 (64.8)
2.	untreated	1250 ± 9 (1.3)	1219 ± 52 (1.0)	N.D.	2262 ± 588 (12.1)
3.	anti-θ-treated	3409 ± 174 (24.4)	2033 ± 60 (9.7)	1545 ± 89 (5.6)	7432 ± 393 (67.4)
4.	NMS-treated	1332 ± 2.2 (2.2)	1295 ± 1.8 (1.8)	1058 ± 63 (0)	2598 ± 220 (15.7)
5.	untreated[c]	1117 ± 34 (1.9)	1162 ± 19 (1.0)	1218 ± 17 (0.6)	1300 ± 425 (1.0)

CBA anti-Balb/c first step cells were treated with either anti-θ serum and complement or NMS and complement and the equivalent to 10^6/raft or $3x10^5$/well untreated cells were added to second step cultures. In Experiment 1, second step cultures were prepared in acrylamide rafts in triplicates. In Experiment 2, second step cultures were prepared with $3x10^5$ CBA lymph node cells and $1.6x10^6$ irradiated Balb/c spleen cells in microtiter trays. Cell recovery was $6x10^6$ cells/ml untreated, $5.4x10^6$ cells/ml NMS treated, $1.5x10^6$ cells/ml anti-θ treated.

[a] mean cpm ± S.D. of six replicate cultures

[b] % specific lysis
spontaneous release: 1124 ± 140 cpm
detergent release: 9876 ± 845 cpm

[c] no responder cells added, irradiated first step cells were cultured with irradiated Balb/c stimulator cells only

Table III. Strain specificity of the suppressor cells

	Responder cells	Irradiated stimulator cells	Irradiated first step culture	anti-(H-2^d) cytotoxic response (culture dilutions) 1/10	1/30	1/60
1.	CBA	Balb/c	-	15069 ± 500[a] (54.5)[b]	9494 ± 320 (30.8)	5719 ± 372 (15.0)
2.	CBA	Balb/c	CBA anti-Balb/c	5981 ± 20 (16.1)	3731 ± 67 (6.7)	3063 ± 102 (3.9)
3.	C3H·SwSn	Balb/c	-	18340 ± 864 (67.9)	11934 ± 322 (41.1)	7563 ± 568 (22.8)
4.	C3H·SwSn	Balb/c	CBA anti-Balb/c	5888 ± 227 (15.7)	3458 ± 44 (5.6)	2956 ± 251 (3.4)
5.	-	-	CBA anti-Balb/c	2205 ± 90 (0.3)	2079 ± 78 (0)	2112 ± 71 (0.1)

Second step cultures were prepared with either 2×10^6 normal CBA (H-2^k) or C3H·SwSn (H-2^b) lymph node cells as responders and 16×10^6 irradiated Balb/c (H-2^d) spleen cells as stimulators with or without 10^6 irradiated CBA anti-Balb/c first step cells. Cells were cultured in polyacrylamide rafts for 5 days.

[a] mean cpm ± S.D.

[b] % specific lysis

spontaneous ^{51}Cr release: 2132 ± 65 cpm

detergent ^{51}Cr release: 23872 ± 693 cpm

suppressor activity by adding to a second step MLC and (b) to assay for direct cytotoxicity. Figure 5a indicates that effective suppression develops at day three and persists through days four and five. A high level of cytotoxic activity was also detected as early as day three (Figure 5b). These observations were consistent with the interpretation that suppressor cells are cytotoxic cells. We decided to test this hypothesis further by examining whether or not suppressor activity could be dissociated from cytotoxic activity by other methods.

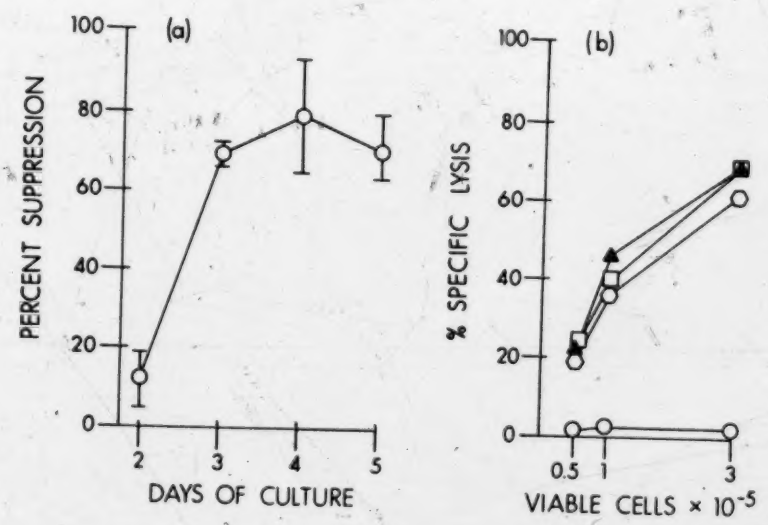

Figure 5. Relationship between time of culture incubation and the development of suppressor cells. First step cultures were prepared with $3x10^6$ CBA lymph node cells and $5x10^6$ irradiated Balb/c spleen cells. After a given period of incubation, the cells were irradiated and divided into two parts: one part was assayed for suppressor activity, Figure 5a, and the second was assayed immediately for cytotoxicity, Figure 5b:
○—○ 2-day first step culture; ▲—▲ 3-day first step culture;
□—□ 4-day first step culture; □—□ 5-day first step culture.
spontaneous release = 710 ± 54 cpm, detergent release = 8305 ± 523 cpm.
(a) % suppression is calculated as:

$$100 = \frac{\text{\% specific lysis of experimental group}}{\text{\% specific lysis of positive control} = (65.4 \pm 2.5\%)}$$

6) The effect of in vivo treatment with hydrocortisone on the subsequent in vitro generation of suppressor cells and cytotoxic cells

First step cultures were prepared with spleen cells from cortisone-treated and normal CBA mice. After three days, the cells were harvested, washed, irradiated and divided into two parts. One part was added to a second MLC to assay suppressor activity and the second was tested for the presence of CTL. Figure 6(a) shows the cytotoxic activity of cells from three-day first step cultures set up with either normal or cortisone-treated responder cells. In general, cultured normal spleen cells had 8-10 times more cytotoxicity than did cultured cortisone-treated spleen cells. The suppressive effect of these two cell populations was measured and the results are shown in Figure 6(b). Irradiated first step cells, in the numbers indicated, derived from cultures of normal spleen cells were able to completely abrogate the response in the second step cultures. In contrast, the first step cells derived from cultures of cortisone-treated spleen were unable to suppress the response at any cell number tested (1-20 $x10^5$ per culture). It is reasonable to compare for suppressive activity of those cell numbers from the two kinds of first step cultures that have comparable levels of cytotoxicity. For example, $1x10^6$ cells from cultures of cortisone-treated mice and $1x10^5$ cells from cultures of normal spleen each exhibited 32-35% cytotoxicity and yet had very different effects on the generation of CTL in the second MLC. Whereas $1x10^5$ first step cells of normal mice completely suppressed the cytotoxic response, $1x10^6$ cells from cultures of cortisone-treated spleen slightly enhanced the response. This observation suggests that suppression is not due to the cytotoxic activity of the cells from first step cultures.

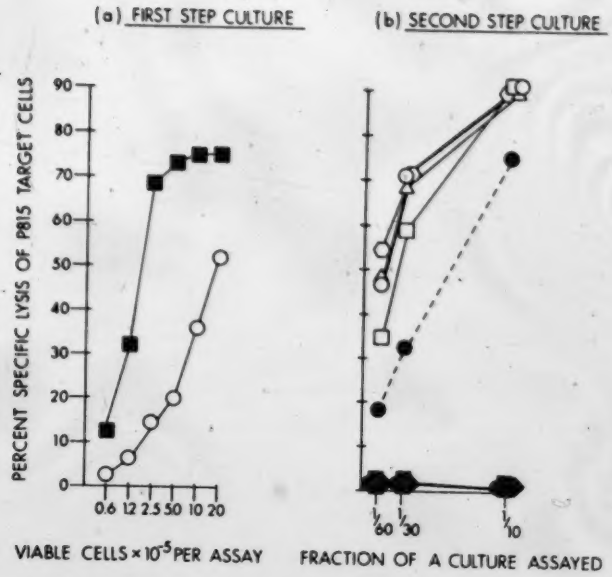

Figure 6. Effect of cortisone treatment on the generation of suppressor cells. First step cultures were prepared with CBA responder spleen cells from cortisone-treated mice (three days after treatment with cortisone) and irradiated Balb/c stimulator spleen cells. Similarly, first step cultures were prepared with CBA responder spleen cells from normal mice and irradiated Balb/c spleen cells. After three days in culture, cells were irradiated and divided into two parts: one part was assayed for cytotoxic activity (a) and the second was assayed for suppressor activity on the generation of CTL (b).

(a) ■—□ normal spleen; ○—○ cortisone-treated spleen.

(b) Second step cultures were prepared with 5×10^5 CBA lymph node responder cells and 16×10^6 irradiated Balb/c spleen stimulator cells in polyacrylamide rafts. These were cultured with first step cells either from normal spleen, closed symbols, or cortisone-treated spleen, open symbols; (□ , ■ 2×10^6; ○ , ● 1×10^6; △ , ▲ 5×10^5; ⬡ , ⬢ 1×10^5). Second step cultures were also prepared without first step cells, ○---○ . spontaneous release = 518 ± 67 cpm, detergent release = 5109 ± 226 cpm.

One could argue that cortisone treatment increases the ability of spleen cells to generate helper cells which then overcome the activity of the suppressor cells generated in the same cultures. This interpretation is supported by the fact that cells from cortisone-treated spleen when cultured generate helper cells which are most effective at the lowest cell number added ($1x10^5$ per culture, Figure 6). Such an interpretation allows us to predict that cells from first step cultures which were prepared from cortisone-treated mice, upon mixing with cells from first step cultures of normal spleen, should reverse the ability of the latter to suppress the generation of CTL. This possibility was tested in the following way. First step cultures were prepared with CBA cells from either cortisone-treated or normal spleen and irradiated Balb/c spleen cells. Cells from each kind of first step culture were tested for (a) the presence of CTL and (b) suppressor cell activity. Different numbers of cells from those cultures of spleen cells derived from cortisone-treated mice were added to a second step MLC, either alone or mixed with a varying number of cells from first step cultures of normal spleen. The results from a representative experiment are shown in Figure 7. First step cells from cultures of cortisone-treated spleen, which had cytotoxic activity, failed to suppress the generation of CTL in the second culture (groups 1.b, c, d). In contrast, a similar number of cells from first step cultures of spleen from normal mice inhibited the generation of the cytotoxic response. Cell numbers of $1x10^6$ and $3x10^5$ from normal first step cultures strongly suppressed the cytotoxic response (groups 2.a, 3.a), whereas cell numbers of $1x10^5$ and $3x10^4$ were weakly suppressive (groups 4.a, 5.a). A lower cell number of $1x10^4$ had no effect on the generation of CTL (data not shown). The addition of first step cells from

cortisone-treated mice, in the numbers indicated, failed to reverse the inhibitory effect, however weak, of normal first step cultures on the generation of CTL (Figure 7, groups 2-5.b, c, d).

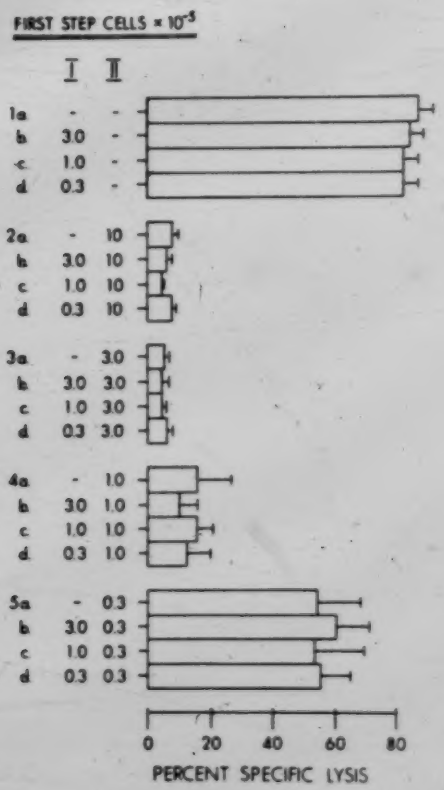

Figure 7. The effect of first step cultures from cortisone-treated mice on suppressive activity of first step cultures from normal spleen. Three day CBA anti-Balb/c first step cells from cortisone-treated mice were added to second step cultures, either alone or mixed with various numbers of CBA anti-Balb/c first step cells from normal spleen. Second step cultures were prepared with $3x10^5$ CBA spleen responder cells and $1x10^6$ irradiated Balb/c stimulator cells in microtiter trays. The values represent the mean ± standard deviation of six replicate cultures. spontaneous release = 537 ± 60 cpm, detergent release = 4427 ± 179 cpm.
I. cortisone-treated spleen x 10^{-5}

Figure 7 cont'd.

II. normal spleen x 10^{-5}. Irradiated controls, cultures with irradiated first step cells and irradiated stimulator cells only, were always negative.

Cytotoxicity of three day first step cultures:

	% specific lysis	
	normal	cortisone-treated
$3x10^5$	75.1	37.0
$1x10^5$	46.7	14.5
$5x10^4$	23.8	7.3

spontaneous release = 733 ± 39 cpm, detergent release - 7207 ± 179 cpm.

7) Mechanism of action of suppressor cells

In the analysis of the specificity of recognition and the nature of interaction of suppressor cells, two broad possibilities can be distinguished. Either (1) physical linkage of determinants recognized by the suppressor cell and the killer precursor cell is required for suppression or (2) physical linkage of determinants is not required. The first possibility predicts that suppression of the generation of cytotoxic cells will be effective only if the determinants recognized by suppressor cells are linked on one physical entity to the determinants recognized by responder cell precursor. The second predicts that suppression will occur if the determinants recognized by the suppressor cells are on a different cell from those recognized by the killer precursors (third party effect). In order to test which of these possibilities is correct, the following experiment was performed. CBA anti-Balb/c suppressor cells from a first step culture were added to second step cultures prepared with CBA spleen responder cells and either Balb/c (H-2^d), C3H·SwSn, or (Balb/c x C3H·SwSn) F1 hybrid spleen stimulator cells. These cultures were assayed for cytotoxic activity against target cells bearing either H-2^d or H-2^b antigens. If the first possibility is correct, anti-Balb/c suppressor cells will inhibit the anti-H-2^b response only in the presence of F1 cells bearing

both the H-2^b amd H-2^d antigens. If the second is correct, then the anti-H-2^b response will be suppressed in both cultures containing a mixture of C3H·SwSn and Balb/c and cultures containing the F1 stimulator cells. These experiments show (Figure 8) that in the presence of irradiated CBA anti-Balb/c suppressor cells the anti-H-2^d response was completely suppressed (Figure 8.a, group 2), whereas the anti-(H-2^b) response was not affected (Figure 8.b, group 2). Furthermore, the cytotoxic response to both H-2^d and H-2^b alloantigens was suppressed in the cultures to which (Balb/c x C3H·SwSn) F1 hybrid stimulator cells were added (group 4, Figures 6a and 6b). On the other hand, only the anti-H-2^d cytotoxic response was efficiently inhibited in those cultures to which a mixture of irradiated Balb/c and C3H·SwSn spleen stimulator cells was added, whereas the anti-H-2^b response was unchanged (group 6, Figures 8a and 8b). These results are in accord with the hypothesis that suppressor cells are highly specific and are effective in suppressing a response only if the target antigen, against which the induction of the cytotoxic response is suppressed, is physically linked to the target antigen for which the suppressor cells are specific.

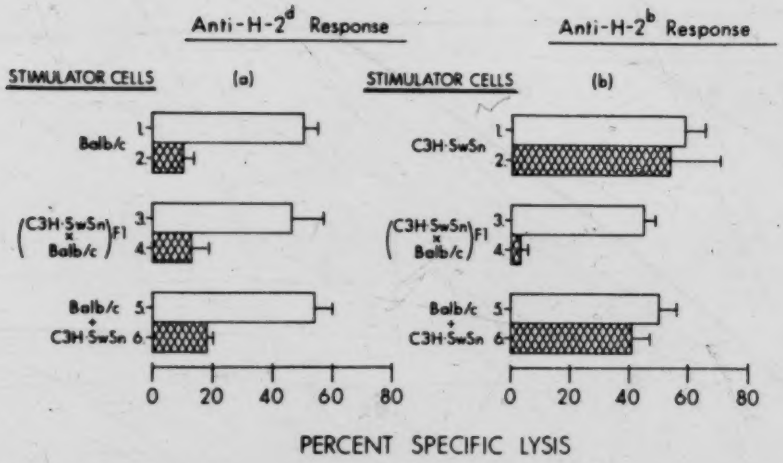

Figure 8. Suppressor cells and responder precursor cells recognize physically linked determinants on the stimulator cells. CBA anti-Balb/c first step cells were added to second step cultures prepared with $3x10^5$ CBA responder cells and $5x10^5$ irradiated spleen stimulator cells of different H-2 haplotypes as indicated. After five days in culture, cytotoxicity was assayed on P815 ($H-2^d$) target cells, Figure 8a, and EL4 ($H-2^b$) target cells, Figure 8b. This experiment was repeated twice in microtiter trays and twice in polyacrylamide rafts and similar results were consistently obtained. ☐ no first step cells added, ▩ first step cells added. P815: spontaneous release = 1193 ± 87 cpm, detergent release = 6653 ± 271 cpm. EL4: spontaneous release = 1679 ± 79 cpm, detergent release = 7851 ± 162 cpm.

Chapter IV. Discussion

The observations presented here show that irradiated CBA anti-Balb/c cells from three days first step cultures, upon transfer to a second MLC, have an immunologically specific suppressive effect on the induction of the anti-Balb/c cytotoxic response. Treatment of these cells with anti-θ and complement abrogates their suppressive effect, confirming the T cell dependence of the inhibitory cells. In addition, anti-Balb/c first step cells can inhibit the induction of CTL by cells bearing a different H-2 haplotype from that of the suppressor cells.

The nature of the suppressive effect of first step cultures on the generation of CTL demands a critical discussion. We have shown that the suppression of the cytotoxic response which we observe at five days of culture is not due to a shift in the time required for the generation of peak cytotoxicity in second step cultures. Fitch et al. (52) have suggested that the suppression of the cytotoxic response could be due to elimination of stimulator cells in the second culture as a result of anti-stimulator cell cytotoxicity generated in first step cultures. The observation that first step cultures have high levels of anti-stimulator cell cytotoxicity (Figure 5) is consistent with this hypothesis. However, studies by Hirano and Nordin (54) on the cortisone sensitivity of suppressor cells led to our observation which indicates that first step cells from cortisone-treated mice and first step cells from normal spleen, at cell numbers exhibiting comparable levels of cytotoxicity, differ markedly in their ability to suppress the induction of CTL (Figure 6). This observation is, at face value, inconsistent with the hypothesis that suppressor cells are cytotoxic T cells. However, maintaining the

assumption that suppressor cells are cytotoxic cells, a different interpretation of this observation is feasible. One could argue that suppressor cells are in fact present in first step cultures from cortisone-treated mice but the expression of the suppressive activity could have been overcome by other effects induced in the same cultures. For example, cortisone treatment in vivo could have altered the balance of lymphoid cell populations within the animal by creating an environment with a high level of helper cell precursors. As a result, the helper effects of cells from first step cultures of cortisone-treated spleen may be dominant over the suppressive effects. If such a dominance were possible in this system, then we would predict that first step cells from cortisone-treated animals should reverse the inhibitory activity of first step cells from cultures of normal spleen. This prediction should apply particularly to situations where the suppressive effect of first step cells of normal spleen is very weak. However, the results shown in Figure 5 indicate that this is not the case. In fact, even in the face of weak suppression, first step cells from cortisone-treated spleen did not influence, in any way, the suppressive effect of first step cultures from normal spleen. Thus we can conclude firstly that first step cultures from cortisone-treated mice contain no elements capable of interfering with suppressor cell activity. Therefore, the lack of suppressive activity in these cultures is due to the absence of suppressor cell precursors in cortisone-treated spleen. Secondly, first step cells from cortisone-treated spleen have a high level of cytotoxicity but no suppressive activity on the generation of CTL. Taken together, these observations support the conclusion that suppressor cells and cytotoxic cells are different cell types.

Further evidence of the fact that suppressor cells and cytotoxic cells

are separate cells comes from studies *in vivo* by Wagner *et al.* (50). In their system, on the basis of cell size, suppressor cell precursors were clearly separable from cytotoxic precursor cells. Furthermore, pre-killer cells did not have a suppressive effect upon the generation of CTL.

The experimental results show that suppressor cells act by linked associative recognition. That is, suppressor T cell-precursor T cell interactions occur by a collaborative mechanism analogous to T-B cell cooperation involving linked recognition of physically associated carrier and haptenic determinants on the stimulator cell. Evidence from a large number of experimental models of both humoral and cell-mediated immunity indicates that both positive (helper) and negative (suppressor) regulatory effects require recognition of at least two determinants on the same antigen (46, 75, 76). One determinant is recognized by a precursor cell and the other is recognized by a regulatory cell or its product. This requirement for an antigen bridge allows regulatory signals to be delivered at short range, for example via cell to cell contact or by short-lived soluble factors, so that only precursor cells binding the appropriate antigens are either specifically induced or suppressed. The consequences of a long-range regulatory signal, on the other hand, are best seen by considering practical possibilities. In the case of positive regulation, if induction were to occur via a long-range factor, then its action would no longer be specific. Under these conditions, one would expect precursor cells which would normally be paralyzed on binding to self components (68) to be activated with the result that autoimmunity would be induced. Similarly, in the case of negative regulation, if a long-range inhibitory signal were to exist, beneficial immune responses would be nonspecifically suppressed. If suppressors act via a short-range

inhibitor, then suppression of antigeneically irrelevant immune responses would be avoided. Bretscher (68), in his theory on immune class regulation, has predicted that suppressor cells must act by the associative recognition of antigen and experimental evidence in support of this prediction has now been reported. Ramshaw et al. (46) and Bullock et al. (76) demonstrated that antigen specific immune T cells were able to suppress the delayed-type hypersensitivity response to another unrelated antigen, provided these antigens were physically linked. Similarly, our observations show that the suppressive effect of irradiated first step cells on the generation of CTL was dependent on the manner in which the two alloantigens were presented. CBA anti-Balb/c first step cells effectively suppressed the response only to Balb/c alloantigens in cultures where mixtures of irradiated Balb/c spleen cells and irradiated C3H·SwSn spleen cells were used as stimulator cells. The cytotoxic response to both alloantigens was suppressed in cultures where irradiated (Balb/c x C3H·SwSn) F1 hybrid spleen cells were used as stimulator cells. This suggests that, to be effective, suppressor cells must specifically recognize determinants that are physically linked to those determinants recognized by responder cell precursors. These results strongly confirm the antigen specificity of suppressor T cells.

Chapter V. Summary

The main conclusion from these experiments is that the antigen specific suppressor T cell previously described by others which inhibits the induction of cytotoxic T lymphocytes is not itself a cytotoxic T cell. Suppressor cells were generated by incubating lymph node or spleen cells from normal CBA mice with x-irradiated Balb/c spleen cells in a first step culture. Three days later, the cells were harvested, washed, x-irradiated, and added to a second mixed lymphocyte culture to measure suppressive activity.

Suppressor cells are not cytotoxic T cells, as cells from first step cultures of cortisone-treated mice displayed high cytotoxicity but had no suppressive effect on the generation of killer cells. It was further demonstrated that these cells failed to influence in any way the suppressive effect, however weak, of cells from first step cultures of normal spleen. These observations are difficult to reconcile with the hypothesis that suppression is due to the killing of the stimulator or the responder cells in the second step culture by cytotoxic T cells. We therefore favor the view that the suppression observed in this system is due to a regulatory signal different from cytotoxicity.

The suppressor T cells described here act by linked associative recognition of antigen. That is, suppressor T cells only inhibit the induction of a precursor cytotoxic T cell in the presence of an antigen to which both the precursor cell and the suppressor cell can bind. In this sense, suppressors act in a manner analogous to helper T cells in T-B cell cooperation; carrier specific helper T cells only enhance an anti-hapten B cell response in the presence of hapten-carrier conjugate. Similarly,

alloantigen a (carrier)-specific suppressor T cells only inhibit allo-antigen b (hapten)-specific cytotoxic responses in the presence of (axb) F1 stimulator cells (hapten-carrier conjugate), not in the presence of a mixture of parental stimulator cells (a + b).

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